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Systems Biology Approach to Transplant Tolerance: Proof of concept experiments using RNA Interference (RNAi) to knock down Hub Genes in Jurkat and HeLa Cells in vitro

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Abstract

Background—Systems biology is gaining importance in studying complex systems such as the functional interconnections of human genes. To investigate the molecular interactions involved in T cell immune responses, we used databases of physical gene-gene interactions to constructed molecular interaction networks (interconnections) with R language algorithms. This helped to identify highly interconnected “hub” genes AT(1)P5C1, IL6ST, PRKCZ, MYC, FOS, JUN and MAPK1. We hypothesized that suppression of these hub genes in the gene network would result in significant phenotypic effects on T cells and examined this in-vitro. The molecular interaction networks were then analyzed and visualized with Cytoscape.

Materials and Methods—Jurkat and HeLa cells were transfected with siRNA for the selected hub genes. Cell proliferation was measured using ATP luminescence and BrdU labeling, which were measured 36, 72 and 96 hours after activation.

Results—Following T cell stimulation, we found a significant decrease in ATP production ($P<0.05$) when the hub genes ATP5C1 and PRKCZ were knocked down using siRNA transfection, whereas no difference in ATP production was observed in siRNA transfected HeLa

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cells. However, HeLa cells showed a significant ($P<0.05$) decrease in cell proliferation when the genes MAPK1, IL6ST, ATP5C1, JUN and FOS were knocked down.

Conclusion—In both Jurkat and HeLa cells, targeted gene knockdown using siRNA showed decreased cell proliferation and ATP production in both Jurkat and HeLa cells. However, Jurkat T cells and HELA cells use different hub genes to regulate activation responses. This experiment provides proof of principle of applying siRNA knockdown of T cell hub genes to evaluate their proliferative capacity and ATP production. This novel concept outlines a systems biology approach to identify hub genes for targeted therapeutics.

Keywords

siRNA Jurkat cells; knockdown; hub genes; systems biology; transplant tolerance

Introduction

T lymphocytes are central to acquired immune responses. They are implicated in both humoral and cell-mediated immune responses and are key mediators of infectious diseases (HIV, HCV), asthma, autoimmune diseases, cancer, and transplant rejection. T cells are subdivided into at least 4 different subsets which play distinct roles in an immune response, including regulatory T cells (T regs), T helper (Th), and effector T cells. T cells stimulate as well as regulate the immune system. Several studies have demonstrated the important role of CD4⁺CD25⁺ T regs for immune homeostasis and the regulation of autoimmunity(2),(3, 4). Moreover, T regs(5), and Th cells(6), play a key role in the maintenance of immune homeostasis and the prevention of autoimmune diseases. In contrast, certain T cell subsets including Th1, Th2, and cytotoxic lymphocytes are important in the activation of immune response. These cells are implicated in allograft injury and rejection(7). Suppression of these T cell subsets is important in treating autoimmune diseases and in prevention of transplant rejection.

Systems biology is gaining importance in identifying how different molecular components interact in complex signaling networks(1, 8). Kirschner describes systems biology as the study of the behavior of complex biological organization and processes in terms of the molecular constituents. It is built on molecular biology, physiology, developmental and evolutionary biology and ecology (9) Integrative methodologies and systems biology can be instrumental in the development of strategies to analyze genomic data to select appropriate treatments of diseases (10, 11)(1, 10, 11). Gene interactions can be visualized through networks by Cytoscape (<http://cytoscape.org/>), in which genes are represented as nodes and the interaction between them as edges (lines) connecting the nodes(12). This can be used to analyze gene-gene interactions involved in allograft injury and rejection, leading to strategies to suppress candidate genes and promote organ tolerance.

The term ‘network’ is used in systems biology to express gene-gene interactions. Networks can either be random (in which most nodes have the same number of connections) or scale free (in which most nodes have few connections and some have a large number of links). Highly connected nodes with a high number of links are called hubs. (13). Scale free networks, unlike random networks, are resistant against random removal of nodes(13).

However, removal of hubs can result in collapse of the network. Our laboratory has constructed molecular interaction networks analyzing modulated genes in a murine asthma model.(14). Our results showed that genes that are peripheral (low connectivity) have a higher level of change in gene expression when compared to hubs and superhubs, which have a lower level of change in gene expression. (12).

Using gene-gene interactions catalogued in the *BIND* (Biomolecular Interaction Network Database)(15), *BioGRID* (Biological General Repository for Interaction Datasets), and *HPRD* (*Human Protein Reference Database*), our laboratory built a network using R language algorithms, showing that the human gene network was scale free. Based on network analysis, we identified highly interconnected genes, thus identifying critical gene hubs (Figure 1 and 2). Using R software (<http://www.r-project.org/>), we selected seven different highly connected genes, ATP5C1, IL6ST, PRKCZ, MYC, MAPK1, FOS, and JUN, as candidates for knock down experiments using siRNA (Table1). These genes were selected because they are hub genes in our Jurkat cell network and have documented roles in cell proliferation, differentiation, and activation responses. Our hypothesis was that knocking down hub genes would cause T cell hyporesponsiveness (16) (17, 18). This is due to their highly connected nature as hub genes. Knocking down these genes could result in collapse of T cell activation genes leading to reduced T cell proliferation and blunting of allograft response. We lipotransfected Jurkat cells (a transformed T cell line) as a model of T cell activation and HeLa cell (immortal cervical cancer cell line) with siRNA and measured markers of cell proliferation. RNA interference has been used to target genes to deactivate them (18). We hypothesized that knocking down these hub genes would result in global cellular effects leading to hyporesponsiveness.

Materials and Methods

Construction of Human gene network

Using publically available gene interactions obtained on BIND (15) for human genome, a network was built using Cytoscape (8), an open bioinformatics software platform for visualizing molecular interaction networks and R statistic language (<http://www.r-project.org/>). ATP5C1, MYC, JUN, IL6ST, PRKCZ, MAPK1 and FOS were identified as hub genes (defined as > 15 connections) in the network and selected for knockdown.

Jurkat cell line

Jurkat cells, an immortalized T cell line, were obtained from ATCC (Virginia, USA) and were grown in GIBCO® RPMI 1640 media with 2 mM L-glutamine (Invitrogen, Carlsbad, U.S.A), 10% Fetal Bovine Serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were incubated with 5% CO₂/95% humidified air at 37°C. Culture medium was replaced every 2-3 days, and cells were passaged to maintain density between 2 x 10⁵ to 1 x 10⁶ cells/ml. Their viability was checked using trypan blue staining. Jurkat cells closely represent T cells which are implicated in allograft rejection. The immortalized cell lines were chosen for this proof of concept experiments due to their ease of availability for invitro experiments (19). They have been widely studied for T cell receptors and cytokine signaling.

HeLa cell line

HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, U.S.A), 10% fetal bovine serum (Clontech), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM L-glutamine, at 37°C and 5% CO₂. The cells were harvested and analyzed under light microscope. Cells were passaged every three days. To evaluate cell viability, HeLa cells were counted and evaluated under the light microscope using the trypan blue stain. HeLa cells were used due to their well known proliferative properties.

Jurkat cells Transfection

Selected siRNA HiPerfect transfection reagents were ordered from Qiagen (Valencia, CA, U.S.A). Transfection was performed according to the supplied protocol (Qiagen, CA, U.S.A). On the day prior to transfection, cells were passaged to a density of 3×10^5 cells per ml and incubated overnight in a spinner flask. On the day of transfection, 2×10^5 cells were added per well to a final volume of 100 µl of RPMI 1640 culture medium with serum and antibiotics in a 24 well plate. 750 ng of siRNA was diluted in 94 µl of RPMI without serum and 3 µl of HiPerfect Transfection Reagent (Qiagen, USA) was then added. Solution was vortexed for 10 seconds and then incubated for 10 minutes at room temperature to allow formation of transfection complexes. Complexes were then added drop-wise to cells and incubated under normal growth conditions for 6 hrs. 400 µl of RPMI 1640 culture medium was then added and the cells were incubated for another 72 hrs. Cells were also transfected with two control siRNA, Allstar negative and Celldeath (Qiagen, CA, USA) and examined at 24 hrs after transfection under fluorescent microscopy to determine transfection efficiency. Allstar negative is a negative control siRNA modified with AlexaFluor488(Invitrogen, Carlsbad, U.S.A) with no known homology to any mammalian gene. Celldeath is a mixture of siRNA targeting genes resulting in cell apoptosis (Invitrogen, Carlsbad, USA).

ATP assay (11)

After 72 hrs of incubation, ATP production was measured as an indicator of cell energy level. Briefly, cells were washed with phosphate buffer solution (PBS) and lysed with lysis buffer. Standard reaction solution was then added consisting of D-luciferin and luciferase according to manufacturer's protocol (ATP determination kit, Invitrogen Carlsbad, U.S.A) and luminescence measured using a liquid scintillation and Luminescence counter (Wallac 1450 MicroBeta TriLux).

HeLa cell line Transfection

HeLa cells (Clontech, Palo Alto, CA) were grown in RPMI medium (Life Technologies, Invitrogen, Bethesda, MD), 10% fetal bovine serum (Clontech, Palo Alto, CA), 100 U/mL penicillin, 100 mg/mL streptomycin, and 1 mM L-glutamine according to Arosio, P et.al. (20). HeLa cells were transfected with 75 ng of siRNA and 4.5µl of HiPerfect, and were incubated for 72 hours at 37°C, 95% RA, 5% CO₂. (Qiagen, California, U.S.A) Viability of cells was analyzed under light microscope using trypan blue staining. To verify transfection efficiency, HeLa cells were transfected under the same conditions with the controls, Allstar negative and Celldeath (Qiagen, Carlsbad, U.S.A).

Cell Proliferation Assay Using BrdU labeling

Cell proliferation was measured using BrdU (Bromodeoxyuridine) following manufacturer's supplied protocol (Roche, Indianapolis, U.S.A.). BrdU, a pyrimidine analogue was added to the cells and cells were labeled for 8 hrs. Briefly, 6×10^4 Jurkat cells were incubated with 250 ng of siRNA and 1 ul of HiPerfect and incubated at 37°C, 95% RA, 5% CO₂ for 36 hrs. 5×10^4 HeLa cells were incubated with 37.5 ng of siRNA and 3 ul of HiPerfect and incubated for 96 hrs. Cells were fixed and DNA denatured by adding FixDenat (Roche, Indianapolis, U.S.A.). Anti-BrdU-POD was then added followed by substrate solution. Absorbance was measured in ELISA reader (Enzyme-linked immunosorbent assay) at 370 nm.

Results

Transfection efficiency

Confirmation of transfection efficiency: Cell culture plates of Jurkat cells transfected with Allstar negative cells served as control. They showed a transfection efficiency of 30-50% compared to 90% transfection efficiency of HeLa cells (Figure 3 A-D).

ATP Production by transfected HeLa cells

HeLa cells were transfected with siRNA to different genes and incubated. ATP production was measured as indirect evidence of cellular proliferation and activity. The results showed a reduction in ATP concentration across the experimental groups. This was not statistically significant, however suppression was observed compared to the control group (Figure 4A).

Proliferation of HeLa cells following transfection

HeLa cells were transfected with siRNA to different genes and incubated. BRDU labeling was done to measure cellular proliferation and activity. The results showed a statistically significant reduction in cellular proliferation of the transfected group compared to controls (Figure 4B).

ATP production by transfected Jurkat cells

Jurkat cells were transfected with siRNA to different genes and incubated. ATP production was measured as indirect evidence of cellular proliferation and activity. The results showed a reduction in ATP concentration in positive control (Celldeath), PRKCZ, and ATP5C1 ($p < 0.05$) groups compared to Allstar negative (Figure 5A). BRDU labeling of Jurkat cells showed proliferative activity of Jurkat cells transfected with MAPK1, MYC, IL6ST, PRKCZ, ATP5C1, JUN and FOS (Figure 5B).

Discussion and Conclusion

This report describes our proof of concept experiments at targeting certain hub genes present in T cells. Silencing these genes to 'induce' allograft tolerance is a new concept that, if successful, will open new horizons to prevent and manage acute and chronic allograft rejection that lead to graft loss. Our lab constructed a molecular interaction network using publically available databases and demonstrated that the human network is scale free. This is consistent with other studies showing that molecular interaction networks in many other

organisms are scale free including *C. elegans*, yeast, and mice. Jeong et al(21) had previously demonstrated in the Yeast protein network that a significantly higher proportion of genes were hubs contained genes that were essential. Unpublished data from our lab involving analysis of publically available protein-protein interactions show that lethal genes in both mice and yeast have a higher connectivity compared to non-lethal genes. Further analysis of genes involved in known disease in the human protein-protein network also demonstrates on an average a higher connectivity compared to non diseased genes. We sought to demonstrate this concept in vitro by selecting several hub genes which are involved in cell cycle progression and apoptosis.

Our proof in concept experiment demonstrated that knockdown of hub genes results in decreased ATP levels in Jurkat T cells compared to the negative control siRNA. In Jurkat cells, there was no difference between control and seven genes in cellular proliferation measured by BRDU. In contrast, in HeLa cells, knockdown of six out of seven genes using siRNA resulted in decreased ATP production and cellular proliferation measured by BRDU. The difference in results between HeLa and Jurkat cells suggests that responses in the Jurkat and HeLa cells are regulated by different hubs. Decreased ATP production by Jurkat cells resulting from siRNA interference and knockdown of hub genes could have therapeutic implications. If confirmed by in vivo studies this strategy could lead to translational studies to prevent and treat organ allograft rejection, in the acute and chronic setting. Lack of suppression of ATP production by Jurkat cells could be related to their in vitro growth potential and characteristics. T cells in vivo, given their increased sensitivity could behave differently and be more susceptible to siRNA induced knockdown.

In the current environment after the completion of the human genome project, over 20,000 genes have been identified. Systems biology offers a unique method of identifying genes of importance based on their connectivity within human molecular interaction networks. We show that down regulation of selected hub genes by siRNA is possible in vitro and could open opportunities to target these genes and offer new targets for drugs or siRNA in vivo. This also demonstrates the feasibility of a systems biology approach in identifying novel genes as potential therapeutic targets.

A limitation of this study includes the inconsistent results between the two cell lines which could be attributed to different transfection efficiencies. Genes which were nodes but not hubs were not studied. In addition, we studied only a limited number of hub genes out of 1400+ or so hub genes identified in our network.

When the cells were treated with shRNA vector construct instead of siRNA lipotransfection, the results of the control experiments using flow cytometry showed that transfection efficacy of Jurkat cells increased to 52% instead of 30%-40% when using siRNA lipotransfection. These results might be due to targeted shRNA effect and more accurate detection of fluorescence using flow cytometry. The shRNA results are preliminary and further experiments are being conducted to confirm Jurkat cells transfection efficacy. In vivo models of solid organ transplantation like heart and liver transplantation will be used to test suppression of T cell activation genes using siRNA technology. T cells will be isolated from the spleen and proliferated and transfected with siRNA for selected hub genes. These cells

will then be injected in animals that receive heart or liver transplant to evaluate allograft survival.

In conclusion, a systems approach can identify potential new gene targets of interests. In this proof of concept experiment, we show that targeting selected hub genes in vitro resulted in decreased cellular proliferation suggesting their importance in human protein-protein network. This technique could have future application in management of certain diseases as well as transplant immunology in the prevention and management of allograft rejection and promoting allograft tolerance. Ability to clinically suppress T cell activation and proliferation using RNAi technology will allow for allograft tolerance and longevity. This will also have a tremendous impact on prevention of chronic allograft rejection, the commonest cause of longterm graft loss. It could also be applied to circumvent ischemia reperfusion injury which is a key physiological response during reperfusion of transplanted organs.

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Figure 1.

Subnetworks of Modulated Genes during T-cell Activation in Jurkat Cells at 8 Hr.

Modulated genes were determined from analyzing activated Jurkat expression values using R language. Measurement of activation of Jurkat cells occurred at various time points: 1 Hr (not shown), 2 Hr (not shown), 4 Hr (not shown), 8 Hr, and 16 Hr (not shown). (Color Code: Modulated genes at 1 Hr = green, 2 Hr = blue, 4 Hr = bright purple, and 8 Hr = red). The activated Jurkat dataset was obtained from the GEO Dataset in Pubmed. Cytoscape v2.6.0 was used to build the subnetwork.

Figure 2.

In a human protein-protein interaction network, there were 11,209 nodes. After arbitrarily defining hubs as nodes with 15 or more edges, 1387 or 12% of all nodes were hubs. Node linkages demonstrated a power law distribution suggesting that the network was scale free

Figure 3. Transfection Efficiency of Jurkat and HeLa cells

A) Jurkat cells in culture under light microscopy. B) Jurkat cells under fluorescent microscopy. Jurkat cells in panel A and B were transfected with Allstar negative, negative control siRNA labeled with AlexaFluor 488, 24 hrs after transfection. Figure C and D shows transfection efficacy of HeLa Cells with Allstar negative, negative control siRNA. A and C are cells in culture, Trypan blue staining under confocal microscope

Figure 4. ATP production and Proliferation of HeLa Cells

Histogram shows ATP production in HeLa cells after incubation with siRNA. Error bars are standard errors with ± 1.96 . 2×10^4 HeLa cells were transfected with 75 ng of siRNA and 4.5ul of HiPerfect, and were incubated for 72 hours at 37°C, 95% FRA, 5% CO₂. Allstar negative is a negative control siRNA with no known homology to human genome Celldeath siRNA is a positive control siRNA targeting genes resulting in apoptosis. The ATP concentration for 5 different siRNA were decreased compared to Allstar negative control. P values were not significant. **Figure 4b:** HeLa cells proliferation aftertransfection using BrdU labelling Histogram showing absorbance of BrdU for controls and 7 tested siRNA in HeLa cells. The experiments were analyzed with 2-tailed Student t-test. $P < 0.05$ for all except for IL6ST and Myc which has p values < 0.15 . Error bars are ± 1.96 standard deviations. 5×10^4 HeLa cells were incubated with 37.5 ng of siRNA and 3 ul of HiPerfect and incubated for 96 hrs.

Figure 5. ATP production and Proliferation of Jurkat Cells

ATP Production of transfected Jurkat cells: Histogram showing ATP production for 7 different siRNAs. Standard Error bars are ± 1.96 standard errors. Jurkat cells were transfected with 750 ng of siRNA and were incubated for 72 hours at 37°C, 95% RA, and 5% CO₂. ATP levels were significantly decreased in the positive control, Celideath, PRKCZ, and ATP5C1 ($p < 0.05$) compared to Allstar negative. **Figure 5b:** Jurkat cell proliferation after transfection using BrdU labelling: Jurkat (6×10^4 cells) + 250 ng of siRNA + 1 ul of HiPerfect incubated for 36 hrs. The histogram shows BRDU labeling for 7 different siRNAs in Jurkat cells. Error bars are ± 1.96 standard errors.

Table 1

Connectivity profile of Knockdown Gene Targets: Table shows number of connectivity of seven different hub genes. The data was generated using R-statistic program. (<http://www.r-project.org/>)

Gene (Gene ID)	Connectivity
MYC (4609)	954
MAPK1 (5594)	160
PRKCZ (5590)	71
ATP5C1 (509)	25
IL6ST (3572)	27
JUN (3725)	334
FOS (2353)	57